

Tissue-Specific Activity of Two Manganese Superoxide Dismutase Promoters in Transgenic Tobacco¹

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In eukaryotes, manganese superoxide dismutase is a nuclear-encoded protein that scavenges superoxide radicals in the mitochondrial matrix. We have isolated two manganese superoxide dismutase genes from *Nicotiana plumbaginifolia* L. and fused the 5' upstream regulatory region of these genes to the β -glucuronidase reporter gene. The two gene fusions displayed a differential tissue specificity in transgenic tobacco (*Nicotiana tabacum*). Promoter activity of the *SodA1* gene fusion was found in the pollen, middle layer, and stomium of anthers, but was usually undetectable in vegetative organs of mature plants. The *SodA2* gene fusion was expressed in the leaves, stems, roots, and flowers. *SodA2* promoter activity was most prominent in the vascular bundles, stomata, axillary buds, pericycle, stomium, and pollen. Histochemical analysis of succinate dehydrogenase activity suggested that the spatial expression of the two gene fusions is generally correlated with mitochondrial respiratory activity.

Aerobic organisms are constantly exposed to the toxic effects of AOS such as superoxide, H_2O_2 , and the hydroxyl radical. Cellular protective mechanisms against these AOS consist of AOS-scavenging enzymes and low-molecular-weight antioxidants such as ascorbate, glutathione, and α -tocopherol (Alscher and Hess, 1993; Foyer and Mullineaux, 1994). The hydroxyl radical is the most toxic for the cell, but it is too reactive to be controlled enzymically (Halliwell and Gutteridge, 1989). Therefore, the enzymic defense against the hydroxyl radical is directed against its precursors, which are the superoxide radical and H_2O_2 ($O_2^{\cdot -} + H_2O_2 \rightarrow O_2 + OH^{\cdot} + OH^{\cdot}$). Superoxide is con-

verted to O_2 and H_2O_2 by SOD, whereas H_2O_2 is removed by catalases and peroxidases (Foyer and Mullineaux, 1994).

Three classes of SODs are distinguished based on their metal cofactor: MnSOD, FeSOD, and Cu/ZnSOD (Bannister et al., 1987). Each of these classes is found in *Nicotiana plumbaginifolia* (Van Camp et al., 1990). They are all nuclear encoded, but the gene products are present in different subcellular compartments (Bowler et al., 1989a; Van Camp et al., 1990; Tsang et al., 1991). MnSOD is located in the mitochondria, FeSOD in the chloroplasts, and Cu/ZnSOD in both the cytosol and the chloroplasts. The occurrence of SOD in peroxisomes has been reported for a number of plant species, but to date has not been investigated in *N. plumbaginifolia* (Bowler et al., 1994). The presence of SOD in different subcellular compartments is required to scavenge the superoxide radical efficiently at its site of formation. Three major sites of superoxide production have been identified in plants: mitochondria, chloroplasts, and cytosol; these correspond to the location of SODs in the cell (Bowler et al., 1994). Additional sources of superoxide may reside in the peroxisomes (Sandalio et al., 1988; del Río et al., 1989) and in the plasma membrane (Doke and Ohashi, 1988).

During mitochondrial respiration, part of the O_2 is reduced by single-electron transfer (Rich and Bonner, 1978), whereas in the chloroplasts electron leakage to O_2 occurs primarily at the reducing site of PSI (Asada and Takahashi, 1987). Therefore, expression of organellar SODs in plants may show a high degree of cell specificity, as determined by the metabolic rate of a cell and its dependence on photophosphorylation or respiration for ATP production. The cellular expression of MnSOD has thus far not been investigated in plants, but a correlation between MnSOD mRNA levels and mitochondrial respiratory activity was suggested by RNA gel blot analysis, which showed high levels of MnSOD mRNA in tissues with elevated respiratory activity or in response to certain stresses (Bowler et al., 1989a; Tsang et al., 1991; Zhu and Scandalios, 1993).

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Abbreviations: AOS, active oxygen species; RT, reverse transcription; SDH, succinate dehydrogenase; SOD, superoxide dismutase.

In this study we have analyzed the cell specificity of two MnSOD promoters in tobacco and the relationship between MnSOD promoter activity and mitochondrial respiration. We have isolated both members of the MnSOD gene family from *N. plumbaginifolia* and determined the transcriptional activity of these genes in different organs and tissues of tobacco by means of reporter gene fusions. This approach has been used previously for other SOD genes, namely a cytosolic Cu/ZnSOD from *N. plumbaginifolia* (Hérouart et al., 1994) and a chloroplastic Cu/ZnSOD from tomato (Kardish et al., 1994), but to our knowledge it has not yet been applied for a mitochondrial SOD in plants. To investigate a correlation between MnSOD promoter activity and mitochondrial respiration, histochemical localization of SDH activity was performed in parallel with the analysis of the reporter gene fusions.

MATERIALS AND METHODS

DNA Gel Blot Hybridizations

DNA was isolated from *Nicotiana plumbaginifolia* L. plants according to the procedure described by Pruitt and Meyerowitz (1986). This DNA was digested with the appropriate restriction enzymes in buffers recommended by the manufacturer (Pharmacia). Digested DNA was separated in 0.8% 40 mM Tris-acetate, 1 mM Tris-acetate/EDTA agarose gels (Sambrook et al., 1989) and transferred to Hybond-N nylon membranes (Amersham) according to the manufacturer's instructions. Probes were prepared by random-primed labeling (Amersham) from a 0.9-kb *HpaI*-*PstI* fragment of pSOD1 (Bowler et al., 1989a) and a 0.5-kb *HpaI*-*SacII* fragment of pGSOD2 (this study). The latter fragment is located upstream from the sequence that encodes the mature protein. Hybridizations were carried out in $3\times$ SSC ($1\times$ SSC = 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 0.1% SDS at 68°C. For higher stringency, the salt concentration in the wash buffer (SSC, 0.5% SDS) was gradually reduced from $3\times$ to $0.1\times$ SSC.

Screening of a Genomic Library in Phage λ Charon 35

Phages (5×10^5) from a *N. plumbaginifolia* genomic library in λ Charon 35 (De Loose et al., 1988) were screened with the *HpaI*-*PstI* fragment of pSOD1 as a probe according to standard techniques (Maniatis et al., 1982). Seven positive phages were obtained and further analyzed.

Construction and Screening of a Genomic Sublibrary

Fifty micrograms of DNA from *N. plumbaginifolia* leaves was digested for 10 h with *HindIII*, and fragments were separated in a 0.8% Tris-acetate/EDTA agarose gel. Fragments between 2.2 and 3.0 kb were eluted from the gel, purified using a GeneClean kit (Bio 101, La Jolla, CA), ligated in the dephosphorylated *HindIII* site of pGEM2 (Promega), and transferred into *Escherichia coli* strain MC1061 by electroporation. Recombinant clones (5×10^4) were screened with the random-primed *HpaI*-*PstI* fragment of pSOD1 as a probe. Three positive clones were isolated and further characterized. Restriction analysis showed that

these clones contained identical plasmids. The plasmid pGSOD1 contained 1126 bp upstream from the initiation codon of *SodA1* and was used for fusion to the GUS coding sequence.

Specific Detection of *SodA1* and *SodA2* Transcripts by RT-PCR Restriction Fragment Analysis

Total RNA was isolated from young leaves, old leaves, roots, and flowers of mature *N. plumbaginifolia* plants as described by Logemann et al. (1987). Eventual DNA contamination was removed by addition of DNase (Life Technologies) and incubation for 15 min at room temperature. The DNase was subsequently inactivated by adding EDTA to a final concentration of 2.5 mM and incubation for 10 min at 65°C. One microgram of DNA-free RNA was used for RT with an antisense primer (5'-CCAGTTCATAACTTTC-CATATGTTC-3') that is completely homologous to both *SodA1* and *SodA2*. cDNA synthesis was performed for 40 min at 42°C using reverse transcriptase (Moloney Murine Leukemia Virus, Life Technologies) according to the manufacturer's instructions. One-fifth of the synthesized cDNA was taken for PCR with the same antisense primer as for RT and 5'-ATTACAATAAIGCCCTTGAACAGC-3' as a sense primer. The sense primer has one mismatch with the *SodA1* and *SodA2* sequences. Amplification was done under different conditions (30–40 cycles, 54–64°C annealing temperature) in a thermal cycler (Techne PHC-3, New Brunswick Scientific, Edison, NJ) using DNA polymerase (AmpliTaq, Perkin-Elmer). Most of these PCR conditions yielded good results. DNA of the selected amplification reaction (30 cycles of 94°C, 1 min; 62°C, 1 min; 72°C, 2 min) was separated in a 0.8% Tris-acetate/EDTA agarose gel. The fragment of the expected size was eluted from the gel, purified using Micropure Separators (Amicon, Beverly, MA), and digested with restriction enzymes. Restriction fragments (50 ng) were separated on polyacrylamide gels (CleanGel, Pharmacia) and visualized by silver staining according to the manufacturer's instructions. As a molecular weight marker, 100 ng of a 50-bp DNA ladder (Boehringer Mannheim) was included.

Construction of Chimeric Genes

To construct pMnSODGUS1A it was necessary to introduce a *NcoI* site into pGSOD1 at the initiation codon. Site-specific mutagenesis was performed by PCR (Landt et al., 1990), using a mutated antisense primer (5'-GGTTCG-TAGTGCCATGGTTGAGATATTC-3') and the T7 primer as the sense primer. The amplified product was double-digested with *NcoI* and *HindIII*, ligated into *NcoI*-*HindIII*-digested pGUS1 (Peleman et al., 1989), and checked by sequence analysis. The chimeric construct was then cloned as a *HindIII*-*XbaI* fragment into the *HindIII*-*XbaI*-digested binary vector pGSV4 (a gift of Dr. J. Botterman, Plant Genetic Systems, Gent, Belgium). Thus, pMnSODGUS1A contained between the T-DNA borders (a) the GUS coding sequence under control of the 5' region (1126 bp) of the *SodA1* gene and fused at the 3' end to the polyadenylation signal of the octopine synthase gene (*ocs*), and (b) the

neomycin phosphotransferase II (*nptII*)-coding sequence under control of the nopaline synthase (*nos*) promoter and with the *ocs* polyadenylation signal. Because the *nos* promoter is in the downstream orientation with respect to the chimeric *gus* construct, the activity from this promoter is unlikely to influence the *gus* expression in MnSODGUS1A transformants.

For the construction of pMnSODGUS1B, the *SodA1* 5' region (1193 bp) was released from pGSOD1 by consecutively digesting it with *SacII*, flushing the open ends with T4 polymerase, and digesting with *HindIII*. This *HindIII*-flushed *SacII* fragment was ligated into *HindIII*-*SmaI*-digested pHW9 (a gift from Dr. J. Botterman, Plant Genetic Systems) to create an in-frame translational fusion located at the start of the MnSOD mature protein. The chimeric construct was transferred into the binary vector pGSV4 as described for MnSODGUS1A.

Because the position of the *SacII* site is conserved between both MnSOD genes, MnSODGUS2 was constructed using a strategy similar to that used for MnSODGUS1B. In the case of *SodA2*, most of the gene is composed of a 3.2-kb *HindIII* fragment that was subcloned from a phage into pGEM2, generating pGSOD2. A 1610-bp *HindIII*-*SacII* fragment from pGSOD2 was fused in frame with the GUS coding sequence and cloned into pHW9. The chimeric gene was subsequently ligated as a *HindIII*-*XbaI* fragment into the *HindIII*-*XbaI*-digested binary vector pGSC1706 (Peleman et al., 1989). The resulting construct, denoted MnSODGUS2, contained between the T-DNA borders, besides the chimeric *gus* gene, also the *nptII*-coding sequence under control of the 35S promoter. The 35S promoter in this construct is downstream in position and orientation to the chimeric *gus* gene.

For the construction of *rbc*-SS-TP-GUS, *Bam*HI sites were introduced by PCR at the ends of the 1.8-kb fragments encoding the GUS protein. After digestion with *Bam*HI, this fragment was inserted into the dephosphorylated *Bam*HI site of pKAH5 (Teeri et al., 1989), creating a fusion of the chloroplastic transit peptide of the small subunit of Rubisco from pea to GUS (*rbc*-SS-TP-GUS). The resultant intermediate expression vector was mobilized to the non-oncogenic Ti plasmid pGV2260 of *Agrobacterium tumefaciens* C58C1Rif^R (Deblaere et al., 1985) by electrotransformation. Co-integration of the intermediate vectors with pGV2260 was confirmed by PCR analysis of total DNA extracted from *Agrobacterium*.

Transformation and Propagation of Plant Material

The various gene fusions were mobilized into *A. tumefaciens* C58C1Rif^R(pGV2260) (Deblaere et al., 1985) with the use of the *E. coli* helper strain HB101(pRK2013) and transferred to leaf discs of *Nicotiana tabacum* cv Petit Havana SR1 according to De Block et al. (1987). Cuttings taken from regenerated plants were transferred to soil and grown under standard greenhouse conditions. S₁ and S₂ plants were obtained by self-pollination of the primary transformants and S₁ plants, respectively.

Subcellular Fractionation

All experiments were carried out at 4°C. Tobacco leaves were cut into small pieces and ground gently with a pestle and mortar in buffer (0.5 M D-sorbitol, 1 mM EDTA-2Na, 0.1% BSA, 2 mM sodium isoascorbate, 50 mM Hepes-KOH, pH 7.2). Crude extracts were obtained by passing homogenized cells through nylon cloths. Filtrates were centrifuged at 2,000g for 2 min to give crude plastidic pellets. Supernatants were centrifuged again at 8,000g for 5 min to give crude mitochondrial pellets. Crude organelle pellets were suspended into the grinding buffer and layered on Percoll (Pharmacia) continuous gradients formed by ultracentrifugation at 50,000g for 40 min. The initial density of the Percoll solution was adjusted to 1.065 g/mL. Subcellular particles in the crude organelle fraction were separated by centrifugation of the gradients at 8,000g for 20 min, and equivalent volumes were collected from the bottom.

Protein Extraction and Determination of Enzyme Activities

Protein extraction and quantitative kinetic analysis of GUS activity was carried out by a fluorimetric assay as described by Breyne et al. (1993). One unit of GUS was defined as the amount of enzyme that produces 1 nmol of product per min at 37°C. Fluorescence values of samples were determined in the presence and absence of a known and similar amount of commercial GUS enzyme (Boehringer Mannheim). The difference between them gives the fluorescence value of the known amount of commercial GUS in each extract, which was used to express the sample values in units of GUS.

Fumarase activities were determined spectrophotometrically by increase of A_{240} ($2530 \text{ M}^{-1} \text{ cm}^{-1}$) as described by Cooper and Beevers (1969). Subcellular fractions prepared by Percoll continuous gradients were used for enzyme reactions carried out at 25°C in 50 mM potassium phosphate buffer (pH 7.2) and 5 mM sodium L-malate as a substrate in a final volume of 600 μL . Cyt *c* oxidase activities were assayed spectrophotometrically by decrease of A_{550} using dithionite-reduced horse heart Cyt *c* as a substrate at 25°C in 50 mM potassium phosphate buffer (pH 7.2) as described by Storrie and Madden (1990). Oxidized Cyt *c* was reduced by dithionite in the reaction buffer and reduced Cyt *c* was diluted 10-fold with the buffer and degassed to keep the ratio A_{550}/A_{565} between 6 and 9. The enzyme reaction was initiated by the addition of reduced Cyt *c* solution (50 μL) to the reaction mixture in a final volume of 500 μL . Protein concentration was determined by the method of Bradford (1976) using Bio-Rad's kit.

Histochemical Analysis of GUS Activity

Sections (80–200 μm) of plant tissue were cut with a vibrotome (Campden Instruments, Sileby, Loughborough, UK) after embedding in 7% agarose. Histochemical localization of GUS activity was performed according to Hérquart et al. (1994).

Histochemical Analysis of SDH Activity

Histochemical determination of SDH activity was performed as described by Gahan and Kalina (1968) with minor modifications. Staining was performed for 15 min to 1 h at 37°C in 100 mM potassium phosphate buffer (pH 7.8) containing 10 mM sodium succinate and 1 mM nitroblue tetrazolium salt. SDH activity is visualized as a purple to black staining. Sodium malonate (100 mM) was used as an inhibitor of SDH activity to confirm the specificity of the assay.

Immunodetection of MnSOD in Leaf Tissue of *N. plumbaginifolia*

Leaf tissue was prepared and sectioned as described by Marrison and Leech (1994). Tissue sections (7 μ m) were placed onto Superfrost Plus microscope slides (BDH Laboratory Supplies, Poole, Dorset, UK) and left to dry on a hot plate overnight at 40°C. Immunolocalization was performed with a 1:100 dilution of MnSOD antiserum (Bowler et al., 1991) according to Marrison and Leech (1994). MnSOD was visualized using fluorescein isothiocyanate isomer I-conjugated goat anti-rabbit antisera (Sigma). The sections were mounted in mounting medium (Vectashield, Vector Laboratories, Burlingame, CA), sealed with nail polish, and viewed using an epifluorescence microscope (Axioskop 50, Zeiss) with filters BP 450–490 for fluorescein isothiocyanate excitation, FT 510 for beam splitting, and LP 520 for emission (filter set 9, Zeiss).

RESULTS

Isolation of Two MnSOD Genes from *N. plumbaginifolia*

To determine the number of MnSOD genes in *N. plumbaginifolia*, DNA gel blot analysis was performed with a MnSOD cDNA probe (Bowler et al., 1989a). In addition to a strongly hybridizing fragment that presumably corresponds to the previously isolated MnSOD sequence (*SodA1*; Bowler et al., 1989a), a second band of weaker intensity was reproducibly observed, suggesting that a second MnSOD gene (*SodA2*) is present in the haploid genome of *N. plumbaginifolia*. This is exemplified for *Bam*HI-restricted DNA in Figure 1A, lane 1.

Subsequently, a genomic library of *N. plumbaginifolia* in phage λ Charon 35 was screened with the MnSOD cDNA probe. Restriction analysis of seven positive candidates showed that they all correspond to one gene, which is different from *SodA1*. A 3.2-kb *Hind*III fragment containing the 5' region of the gene was subcloned. Sequence analysis confirmed that the isolated gene does not correspond to *SodA1* cDNA; therefore, this gene was designated *SodA2*. Southern blot analysis with a gene-specific *SodA2* probe showed that the *SodA2* gene is located on an 11.5-kb *Bam*HI fragment, which corresponds to the weakly hybridizing band previously observed with the *SodA1* cDNA probe (Fig. 1A, lane 2).

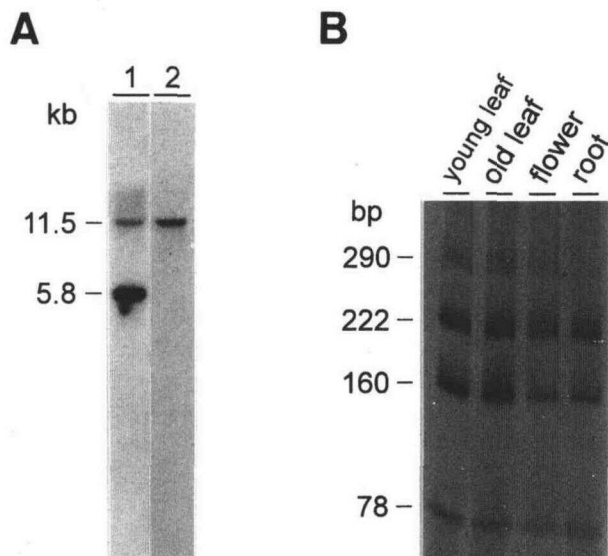


Figure 1. A, DNA gel blot analysis of *Bam*HI-digested DNA from *N. plumbaginifolia*, probed with *SodA1* cDNA *Hpa*I-*Pst*I fragment (complete coding sequence) (lane 1) and probed with *SodA2* *Hpa*I-*Sac*I fragment (5' region) (lane 2). B, RT-PCR restriction fragment analysis of the relative abundance of *SodA1* and *SodA2* mRNA in different organs of *N. plumbaginifolia*. RT-PCR products were digested with *Cfo*I, separated by PAGE, and visualized by silver staining. The sizes of the fragments (*SodA1*: 78, 160, and 222 bp; *SodA2*: 160 and 290 bp) are indicated on the left.

Because the *SodA1* gene was apparently not represented in the genomic phage library, a genomic sublibrary in pGEM2 was constructed that contains 2.2- to 3.0-kb *Hind*III fragments of *N. plumbaginifolia* DNA (see "Materials and Methods"). The 5' region of *SodA1* gene is located on a 2.6-kb *Hind*III fragment (data not shown) and thus should be present in this genomic sublibrary. Screening with a *SodA1* cDNA probe yielded three positive clones with a 2.6-kb insert and a similar restriction pattern. Sequence analysis confirmed that the isolated gene corresponds to *SodA1*.

Specific Detection of *SodA1* and *SodA2* Transcripts by RT-PCR Restriction Fragment Analysis

The relative amounts of *SodA1* and *SodA2* mRNA in different plant organs were determined by a semiquantitative RT-PCR approach. This strategy was chosen because RNA gel blot hybridizations with *SodA1*- and *SodA2*-specific probes did not give satisfactory results. Using primers that are highly homologous to both *SodA1* and *SodA2*, *SodA* transcripts were specifically amplified by RT-PCR. RT-PCR products from both *SodA* genes were discriminated by restriction fragment analysis. Digestion with *Cfo*I gave fragments of 78, 160, and 222 bp in the case of *SodA1*, and fragments of 160 and 290 bp for *SodA2*. Figure 1B shows that the RT-PCR product of *SodA1* is more abundant than that of *SodA2* in all organs analyzed.

Construction of Chimeric Genes Containing the 5' Region of the MnSOD Genes Fused to the Coding Region of the *uidA* Gene and Transfer to Tobacco

To identify the tissue specificity of MnSOD expression, gene fusions were made between the 5' region of the MnSOD genes and the coding region of the GUS gene (*uidA*) of *E. coli* (Fig. 2). In the case of the *SodA1* gene, two translational fusions with the GUS coding sequences were constructed. A translational fusion at the initiation codon was made after introduction of an *NcoI* restriction site in the *SodA1* gene using site-directed mutagenesis by PCR (MnSODGUS1A). A second fusion was made at the start of the mature MnSOD protein (MnSODGUS1B).

Since both MnSODGUS1 constructs gave similar expression patterns (see below), only the translational fusion that contains the mitochondrial transit sequence was constructed for the *SodA2* gene (MnSODGUS2). The three chimeric constructs were cloned in a binary vector and transferred by triparental crossing into *Agrobacterium tumefaciens*.

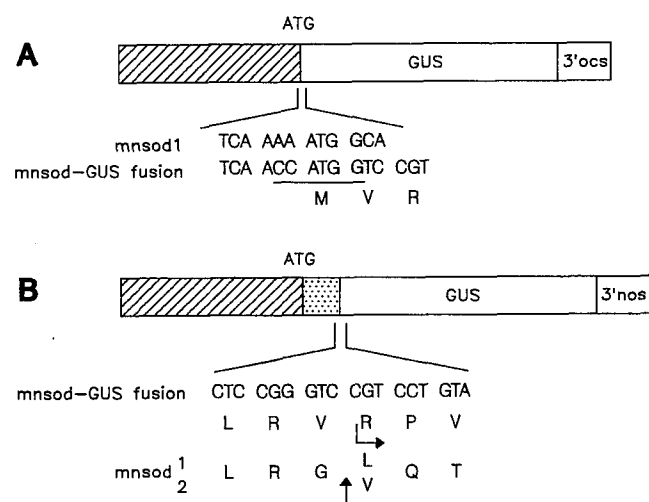


Figure 2. Structure of *SodaA*-GUS fusions. *SodaA* noncoding region is indicated by a hatched box, *SodaA* sequence coding for a mitochondrial transit peptide is indicated by a dotted box. 3' nos and 3' ocs are the polyadenylation sites of the nopaline synthase gene and the octopine synthase gene. ATG, Initiation codon. A, Translational fusion at the MnSOD1 initiation codon. The construct MnSODGUS1A contains a 1130-bp *HindIII*-*NcoI* fragment from *SodA1* fused to the coding region of *uidA*. The *NcoI* restriction site (underlined) in *SodA1* was created by PCR with a mutated 3' primer. The original *SodA1* sequence and the MnSODGUS1A sequence around the initiation codon are indicated. B, Translational fusion at the start of the mature MnSOD protein. The constructs MnSODGUS1B and MnSODGUS2 contain, respectively, a 1193-bp *HindIII*-*SacII* fragment from *SodA1* and a 1610-bp *HindIII*-*SacII* fragment from *SodA2* fused to the coding region of the GUS gene (*uidA*). In these constructs GUS is expressed with a 21-amino acid extension at the NH₂ terminus that has the properties of a mitochondrial transit peptide. The sequence and the corresponding amino acids at the fusion site are shown. The amino terminus of GUS is indicated by a horizontal arrow. The amino acid sequences of MnSOD1 and MnSOD2 at the processing site are shown at the bottom of the figure. The processing site is indicated by a vertical arrow. For details of the constructions, see "Materials and Methods."

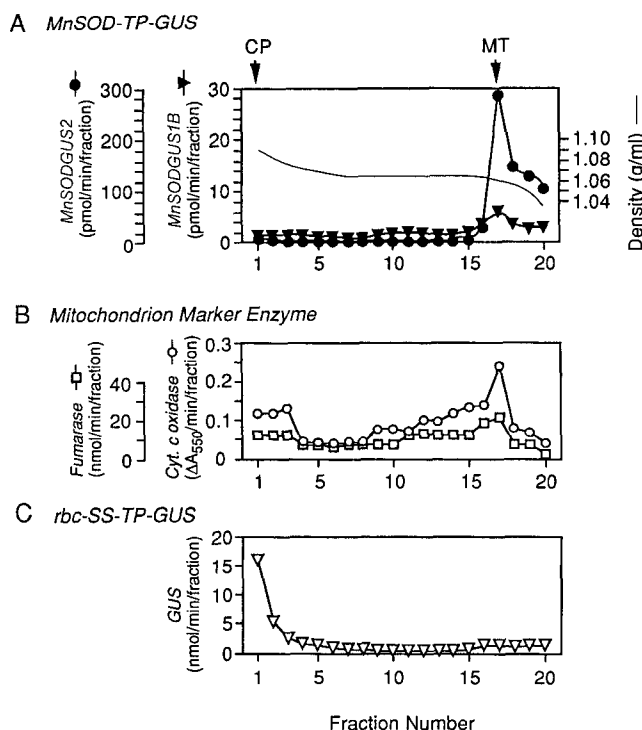


Figure 3. Subcellular distribution of GUS activities in MnSODGUS1B and MnSODGUS2 plants (A), of fumarase and Cyt c oxidase activities (B), and of GUS activity in *rbc-SS-TP-GUS* plants (C). CP, Chloroplast; MT, mitochondria.

faciens. Subsequently, leaf discs of *Nicotiana tabacum* cv Petit Havana SR1 were infected and transformed callus cells were selected on kanamycin-containing medium. Histochemical analysis of GUS activity was performed on leaf, stem, and root tissue of regenerated plantlets to select transgenic plants for cultivation in the greenhouse. No aberrant expression patterns were observed within the primary transformants of each construct. The results presented here for *SodA1* and *SodA2* were mostly obtained with self-fertilized T₁ and T₂ plants of at least five independent transformants. MnSODGUS1A and MnSODGUS1B plants gave a similar expression pattern (data not shown); therefore, only results obtained with MnSODGUS1B plants are presented here.

Targeting of GUS to Leaf Mitochondria

The transgenic tobacco lines MnSODGUS1B and MnSODGUS2 that express the GUS coding sequence fused to the MnSOD transit peptide were analyzed for mitochondrial targeting of the GUS protein. The subcellular location of the GUS protein was determined by subcellular fractionation using Percoll-continuous gradients formed by ultracentrifugation to separate chloroplasts, mitochondria, and other particles having densities intermediate between these two organelles. All subcellular fractions were assayed for GUS (Fig. 3A) and for fumarase and Cyt c oxidase activities (Fig. 3B) as mitochondrial marker enzymes of matrix and membranes, respectively. Transgenic tobacco plants that

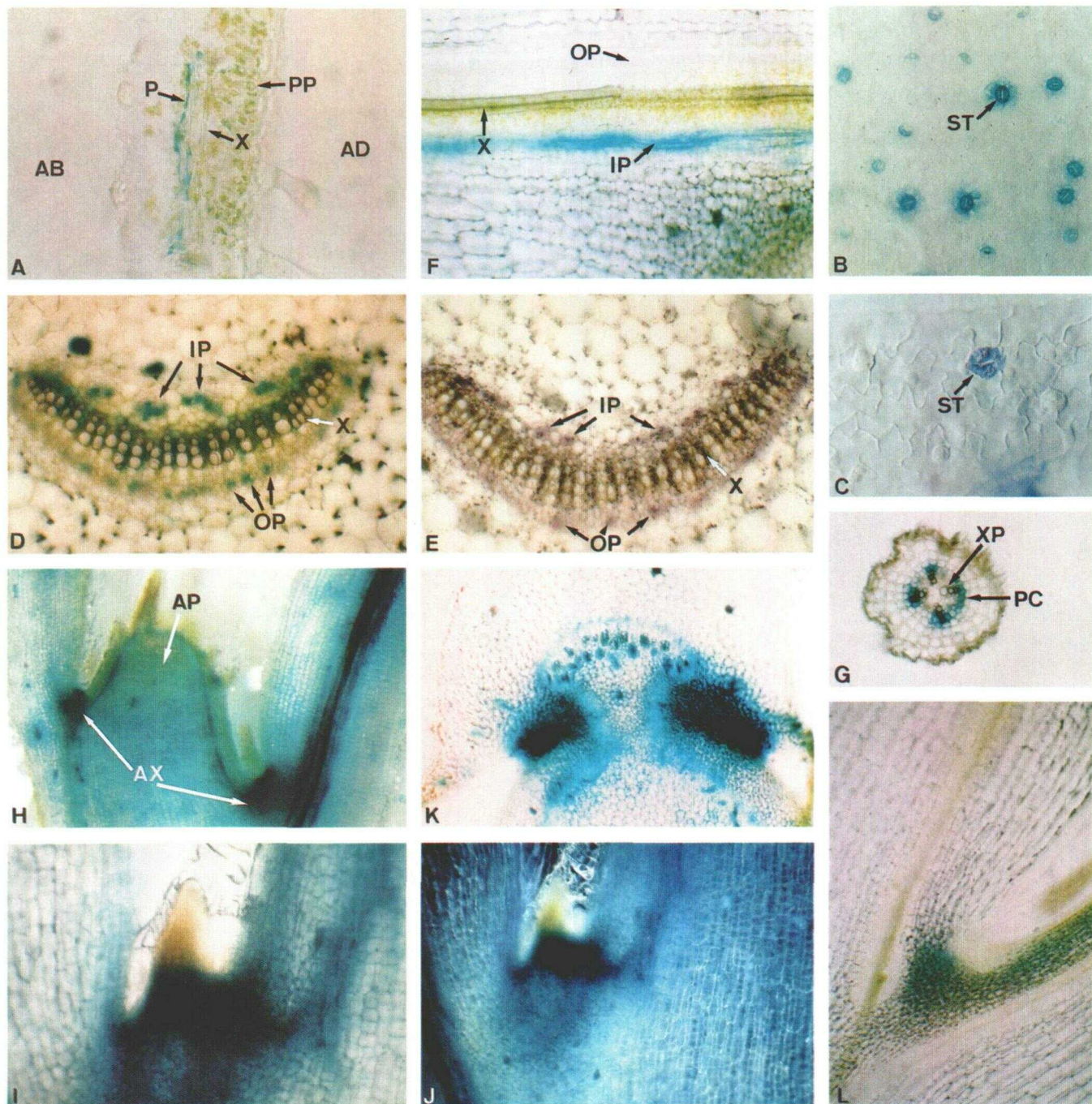


Figure 4. Histochemical localization of GUS and SDH activity in vegetative tissues of MnSODGUS1B (L) and MnSODGUS2 (A–K) plants. A, Leaf, cross-section (GUS). B, Leaf surface (GUS). C, Leaf surface (SDH). D, Petiole, cross-section (GUS). E, Petiole, cross-section (SDH). F, Stem, longitudinal section (GUS). G, Root, cross-section (GUS). H, Apex with leaf primordia, longitudinal section (GUS). I, Axillary bud, longitudinal section (GUS). J, Axillary bud, longitudinal section (SDH). K, Axillary bud, cross-section (GUS). L, Axillary bud, longitudinal section (GUS). AB, Abaxial surface; AD, adaxial surface; AP, apex; AX, axillary bud; IP, inner phloem; OP, outer phloem; P, phloem; PC, pericycle; PP, palisade parenchyma; ST, stoma; X, xylem; XP, xylem poles.

express the *rbc*-SS-TP-GUS fusion protein under control of the cauliflower mosaic virus 35S promoter were used as an indicator of chloroplast targeting (Fig. 3C).

The absolute coincidence of localization of GUS with fumarase and Cyt *c* oxidase in fractions of Percoll gradients unambiguously demonstrates the accumulation of

GUS protein in the mitochondria of MnSODGUS1B and MnSODGUS2 plants. The large difference in GUS enzyme activities in the isolated mitochondria from MnSODGUS1B and MnSODGUS2 plants is due to differential activity of the two MnSOD promoters in green leaves (see below).

Activity of the Chimeric *SodA*::GUS Genes in Vegetative Tissues

Histochemical analysis of GUS activity in soil-grown MnSODGUS1B plants showed almost no expression of the *SodA1*::GUS chimeric gene in mature leaves. Only in a few cases could very faint staining be observed in the vascular tissue. In mature leaves of MnSODGUS2 plants, GUS activity was found in the vascular tissue (Fig. 4A) and in stomatal guard cells (Fig. 4B). Moreover, levels of GUS activity were much higher than in MnSODGUS1B plants. Using fluorimetric GUS assays, it was estimated that for the *SodA2*::GUS chimeric gene activity levels in leaves are as much as 500-fold higher than for the *SodA1*::GUS construct (1 unit versus 2 milliunits GUS per mg protein).

Staining of cross-sections through the petiole of MnSODGUS2 plants indicated that the vascular expression was localized mainly in phloem cells and in the cells adjacent to the xylem vessels (Fig. 4D). Whereas in the petiole no clear difference was observed between expression levels in inner and outer phloem, GUS expression in the stem was most prominent in the inner phloem (Fig. 4F). In roots, staining was seen mainly in the pericycle cells that are adjacent to the xylem poles (Fig. 4G). GUS activity of the *SodA1* chimeric construct was absent or rarely detectable in stem and root (data not shown).

In the growing parts of MnSODGUS2 plants, histochemically detectable GUS activity was highest in newly formed axils near the apex (Fig. 4H) and in axillary buds with leaf primordia (Fig. 4, I and K). Figure 4I shows that the strongest staining is observed at the base of the emerging bud. Some staining in the axillary bud is also observed for the *SodA1*::GUS chimeric gene (Fig. 4L).

Using immunofluorescent techniques, we also investigated the presence of MnSOD protein in different cell types of the leaf. Labeling with MnSOD antibody and fluorescein isothiocyanate isomer I was most pronounced in vascular tissue (Fig. 5) and stomata (data not shown) and hardly detectable in mesophyll or palisade parenchyma cells. Thus, the expression pattern of immunodetectable MnSOD corroborates the histochemical localization of GUS activity in the leaf.

Comparison with SDH Activity

Respiratory activity is thought to be the major source of superoxide radicals in mitochondria (Rich and Bonner, 1978). Therefore, cells or tissues with high respiratory activity may require the highest expression of MnSOD, provided that the relative amount of oxygen that is reduced by monovalent electron addition during respiration is constant in different tissues. To investigate the relation between MnSOD expression and mitochondrial respiration, SDH activity was analyzed in different tissues. In the green parts of tobacco, the highest levels of SDH activity were found in the stomata (Fig. 4C), in the vascular tissue (Fig. 4E), and in the axillary zone (Fig. 4J). In these tissues SDH and GUS activity are localized within the same cells. However, SDH activity was also very high in meristematic cells, particularly in the root

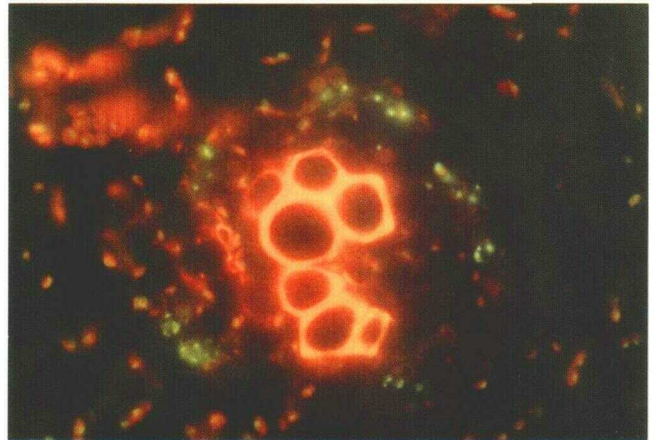


Figure 5. Immunolocalization of MnSOD protein in tobacco leaf sections with MnSOD antibody and fluorescein isothiocyanate isomer I, showing specific expression in vascular tissue. Immunofluorescence is not equally distributed over the cytoplasm, but is confined to cellular bodies, which, in view of the mitochondrial location of MnSOD, may tentatively be identified as mitochondria.

apex, which is in contrast to GUS activity (data not shown).

GUS and SDH Activity in the Flower Parts of Tobacco

In sepals, petals, carpels, and style, GUS activity was localized in the vascular tissue in MnSODGUS2 plants and was absent in MnSODGUS1B plants. Also, SDH activity was seen mainly in the vascular tissue of these organs, but it was also found in ovules (Fig. 6, A–C). In anthers, expression of the MnSOD chimeric genes changed during flower development. In flower buds of 8 to 10 mm, the *SodA2*::GUS chimeric construct was expressed in the central vascular bundle and in a cluster of cells between the stomium and the connective tissue (Fig. 6D). In later stages (flower bud > 12 mm), GUS activity near the stomium disappeared due to degeneration of this tissue, but at the same time GUS activity was developed by the maturing pollen grains (Fig. 6E). Finally, staining in the vascular tissue declined concurrently with the degradation of the connective tissue, whereas GUS activity in pollen was maintained at least until pollen dehiscence (Fig. 6F).

Contrary to mature vegetative tissues, the *SodA1*::GUS chimeric gene was expressed to well detectable levels in anthers. The observed expression pattern was different from that of the *SodA2*::GUS construct. In anthers of 8- to 10-mm buds, GUS activity was seen between the tapetum and the connective tissue (Fig. 6G). This tissue most likely corresponds to the middle layer of the microsporangium wall. In addition, staining was sometimes observed in the outer cells of the stomium (Fig. 6G). Staining in these tissues declined during further anther development, whereas GUS activity in pollen appeared when flower buds reached approximately 12 mm, which is similar to *SodA2* (Fig. 6H). Expression of the *SodA1*::GUS chimeric gene was also observed in mature pollen (Fig. 6I).

SDH activity was seen in all anther tissues that display expression of *SodA1*::GUS and/or *SodA2*::GUS constructs,

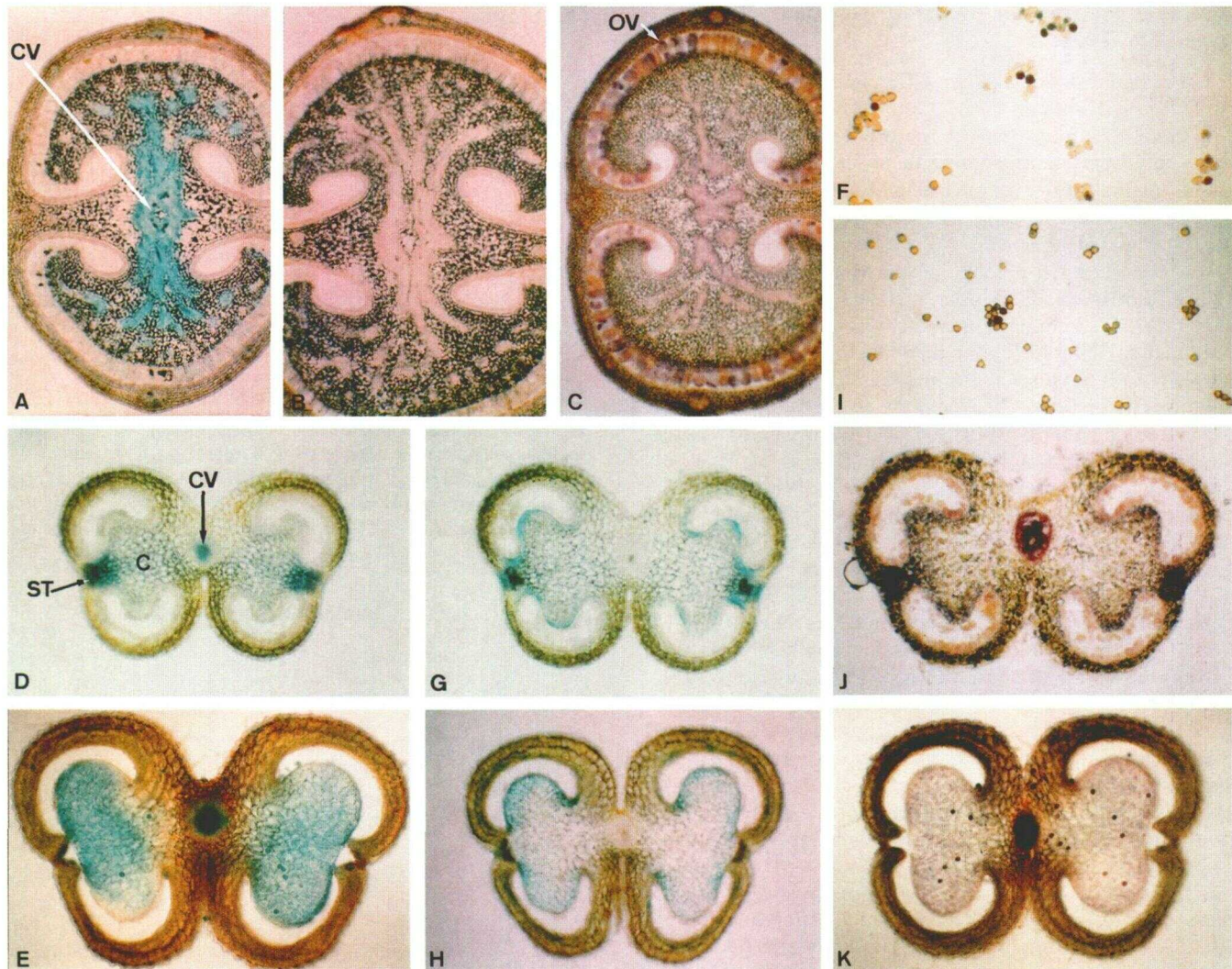


Figure 6. Histochemical localization of SDH activity (C, J, and K) and GUS activity in MnSODGUS2 (A, D-F) and MnSODGUS1B (B, G-I) plants. A-C, Ovary, cross-section. D, G, and J, Anther of 8- to 10-mm bud, cross-section. E, H, and K, Anther of 12- to 14-mm bud, cross-section. F and I, Mature pollen. C, Connective tissue; CV, central vascular bundle; OV, ovule; ST, stomium.

with the exception of the outer cells of the stomium (Fig. 6, J and K).

The Expression of MnSODGUS1B and MnSODGUS2 Constructs Is Not Influenced by a Decreased Level of MnSOD in the Mitochondria

MnSOD expression is assumed to respond to the amount of oxidative stress that is imposed on the mitochondria. To

investigate whether the transcriptional activity of the *SodA* promoters is determined by the needs for MnSOD activity in the mitochondria, crosses were made of both *SodA* promoter-GUS fusions with a transgenic *N. tabacum* cv Petit Havana SR1, transformed with a MnSOD antisense construct under control of the cauliflower mosaic virus 35S promoter. The latter line contains a single insertion locus of the antisense construct and is homozygous at this locus.

Table 1. GUS activities per mg protein in 20-d-old seedlings from MnSODGUS1B and MnSODGUS2 plants crossed either with wild-type SR1 or with a MnSOD antisense line (MnSODAS)

Paraquat was added to the medium to a final concentration of 50 μ M, 17 h prior to harvesting. Water was added in the nontreated samples. Each value is the mean of two samples from independent crosses. Each sample is a pool of about 50 seedlings. All values have been doubled to compensate for the absence of the GUS construct in half of the seedlings.

	MnSODGUS1B × SR1	MnSODGUS1B × MnSODAS	MnSODGUS2 × SR1	MnSODGUS2 × MnSODAS
Nontreated	2.3 milliunits	1.7 milliunits	0.8 unit	1.0 unit
Paraquat-treated	1.9 milliunits	1.9 milliunits	1.0 unit	0.8 unit

The residual MnSOD activity in leaves from this line is less than 10% of the level in untransformed SR1 (W. Van Camp, unpublished data). The MnSODGUS1B and MnSODGUS2 lines used for this cross have a single insertion locus of the transgene and are heterozygous at this locus. Since both the promoter-GUS fusion constructs and the MnSOD antisense constructs contain the *nptII* gene as selectable marker, no selection can be performed for the presence of both foreign genes in the progeny. Thus, whereas all the progeny will express the MnSOD antisense gene, only half will contain the promoter-GUS construct. As controls, back-crosses of the MnSODGUS1B and MnSODGUS2 lines to wild-type SR1 were performed.

The effect of MnSOD suppression on the activity of the promoter-GUS fusions was analyzed in 20-d-old seedlings grown on agar medium (one-half Murashige and Skoog salts, 1% Suc, 0.8% agar, pH 5.7), to which paraquat (50 μ M final concentration) or water was added 17 h prior to harvesting. As shown in Table I, promoter activities of the MnSODGUS1B and MnSODGUS2 constructs were not significantly different in plants with normal or reduced levels of MnSOD; neither did the paraquat treatment cause any induction of the *SodA1* and *SodA2* promoters in the chimeric constructs.

DISCUSSION

In mature leaves of *N. plumbaginifolia* and *N. tabacum*, MnSOD activity levels are generally much lower than those of FeSOD or Cu/ZnSOD (Van Camp et al., 1990, 1994), which suggests that mitochondrial superoxide production in leaves is marginal compared with that in the cytosol and chloroplasts. Similarly, it was observed that mitochondrial overproduction of SOD in transgenic tobacco was less effective than chloroplastic overproduction in protecting plants against leaf damage caused by paraquat (Bowler et al., 1991) or ozone (Van Camp et al., 1994). However, this does not exclude the fact that MnSOD could play a crucial role in the oxidative stress response in tissues other than leaves, or in other developmental stages.

To obtain a more complete picture of MnSOD expression in different tissues, it was first necessary to characterize the MnSOD gene family. We have identified two MnSOD-encoding genes in *N. plumbaginifolia*, denoted *SodA1* and *SodA2*, and we have isolated the 5' ends of both genes. The two MnSOD genes show extensive homology in the coding sequence (88% at the amino acid level), but only two stretches of homology were found in the 5' upstream region. In the 5' untranslated leader, a sequence of 38 nucleotides (shortest sequence, not counting gaps) was found that shows 71% sequence identity (taking gaps as mismatches). A second stretch of 54 nucleotides is 79% identical between both *SodA* genes. This is located at positions -826 to -773 (distance to the translation start) in *SodA1*, and at positions -911 to -857 in *SodA2*.

By RT-PCR restriction fragment analysis, it was estimated that *SodA1* expression in leaves, flowers, and roots was stronger than that of *SodA2*. The tissue specificity of MnSOD expression was further investigated in transgenic

tobacco containing gene fusions of the 5' upstream regions of *SodA1* and *SodA2* with the GUS reporter gene. Two translational fusions have been made for the *SodA1* gene, one at the initiation codon and a second at the start of the mature protein. In the latter construct, a 21-amino acid terminal extension was fused to the reporter protein GUS that has the characteristics of a mitochondrial transit peptide (Bowler et al., 1989b). Using subcellular fractionation techniques, we demonstrated that the MnSOD1 amino-terminal extension can target GUS to mitochondria and that the GUS protein retained its enzymic activity after transport through the mitochondrial membrane. The fact that no significant GUS activity was found in the cytosolic fraction suggests that most of the GUS fusion protein was imported into mitochondria. This result is in accord with the data from Schmitz and Lonsdale (1989), who showed that a fusion of a yeast mitochondrial transit sequence to GUS is efficiently imported to plant mitochondria. Both translational fusions gave the same spatial expression pattern, suggesting that the MnSOD transit peptide or the mitochondrial import do not function as posttranslational control mechanisms of expression. For this reason, it was decided to make only one chimeric construct for the *SodA2* gene that produces a fusion protein of the MnSOD2 transit peptide and GUS. As with MnSOD1, the amino-terminal part of MnSOD2 was active in targeting GUS to the mitochondria.

SodA1::GUS and *SodA2*::GUS promoter-GUS fusions displayed distinct patterns of expression in transgenic tobacco. In leaves, *SodA2* promoter activity was as much as 500-fold higher than that of *SodA1*. Expression of the *SodA2*::GUS construct was strongest in phloem cells and stomatal guard cells. In the case of the *SodA1*::GUS construct, expression was barely detectable by histochemical GUS staining, but, when observed, it was always found in the same tissues where the *SodA2* gene was expressed. The large difference in promoter activity of both *SodA* genes in leaves is at variance with the RT-PCR data on relative *SodA1* and *SodA2* mRNA abundance. The most probable explanation is that some important enhancer sequences are not contained in the *SodA1*::GUS construct. Regulatory elements for spatial expression of plant genes are usually located in the first 500 bp upstream of the transcription initiation site, but enhancer sequences are often found at more distal positions. To confirm the results on the spatial expression of MnSOD in leaves, we have localized MnSOD protein on leaf sections by immunodetection with MnSOD-specific antibodies. MnSOD protein was most abundant in phloem and stomata, which is in accord with the expression data obtained with the promoter-GUS constructs.

SodA1::GUS and *SodA2*::GUS fusions showed distinct spatial expression patterns in anthers. *SodA1*::GUS expression was observed without concurrent expression of the *SodA2* gene in the stomium and the middle layer between connective tissue and tapetum. Although several mRNAs have been identified that are present either exclusively or at elevated levels in tobacco anthers (Koltunow et al., 1990), to our knowledge *SodA1* is the first example of a gene that is highly transcribed in the middle layer of the anther. Only the

SodA2::GUS construct showed activity in a cluster of cells between the connective tissue and the stomium. Preferential expression in this cluster has also been observed for a thiol endopeptidase from tobacco (Koltunow et al., 1990). Pollen was the only part of the anther in which both promoter::GUS fusions were expressed simultaneously. It has been proposed that simultaneous expression of several MnSOD genes would occur only in tissues that need to cope with a very high rate of superoxide production in the mitochondria (Zhu and Scandalios, 1993). When applied to anthers, this hypothesis would predict that mitochondrial superoxide production is most elevated in pollen, which is not unlikely, considering the high metabolic activity of pollen.

To investigate whether the tissue-specific patterns of *SodA* promoter activity would correlate with mitochondrial respiratory activity, histochemical analysis of SDH activity was performed. At present, no methods exist that allow the measurement of in situ mitochondrial activity at the cellular level. Therefore, we decided to monitor SDH activity for which a histochemical activity assay has been described (Gahan and Kalina, 1968). Besides NADH dehydrogenase, SDH is the second key entrance point for electrons in the mitochondrial respiratory electron transport chain. Consequently, SDH activity may give a fairly good idea of the mitochondrial respiratory activity of a cell. SDH activity coincided with *SodA1* and *SodA2* promoter activity in the green parts of the plant and in pollen, suggesting that respiratory electron transport constitutes a major source of superoxide in mitochondria of these tissues. In the root apex and in ovules, SDH activity was seen without (histochemically) detectable GUS activity. The reason for this is not clear, but it may reflect a reduced rate of superoxide formation from the respiratory electron transport chain in these cells. Tissues with densely packed cells, such as the root apex, generally have a lower O₂ tension and thus may produce less superoxide.

CONCLUSIONS

We have studied the tissue-specific expression of two *SodA* promoters in transgenic tobacco. GUS activity of the *SodA::GUS* constructs in leaves co-localized with immunodetectable MnSOD protein, indicating that the regulatory elements determining the spatial expression of MnSOD are located within the 5' upstream regions that were used for the reporter gene constructs. Comparison of GUS activities with mRNA levels revealed that regulatory elements for paraquat induction and enhancer elements for *SodA1* expression in leaves, stems, and roots are not contained within these constructs. MnSOD expression was detected in only a few cell types of the leaf, both by histochemical GUS staining and by immunolocalization. Considering that neither method is quantitative, it is nonetheless surprising that a highly confined expression pattern is observed for a protein that is considered to have a "housekeeping" function. Expression in anthers was even more complex, since *SodA1::GUS* and *SodA2::GUS* constructs were active in distinct cell types and only during specific developmental stages. *SodA* expression in *N. plumbaginifolia* is induced by Suc, and this induction was shown to correlate with an increase in respiratory activity (measured as Cyt oxidase activity; Bowler et al., 1989a). We

have shown now that the tissue-specific pattern of *SodA* expression in most cells also coincides with high mitochondrial respiratory activity (detected histochemically as SDH activity). It is likely that cells with high mitochondrial respiratory activity also require high MnSOD levels for their defense, since respiratory electron transport is able to reduce oxygen monovalently to superoxide (Rich and Bonner, 1978; Turrens and Boveris, 1980). Our data are in accord with this hypothesis and present a first step toward the identification of plant tissues that are particularly prone to oxidative stress in the mitochondria.

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